



Research Article

Production, Egg Quality, and Leukocyte Responses of Late-Laying Hens Receiving Graded Concentrations of Rough Lemon (*Citrus jambhiri*) Juice During Cyclic Heat Exposure-A Preliminary Concentration Comparison Within a Single Heated Environment

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Abstract | Rough lemon juice (RLJ) contains bioactive constituents that warrant evaluation as a locally available drinking-water supplement for laying hens exposed to high ambient temperature. This preliminary study evaluated production performance, selected egg-quality characteristics, and leukocyte responses to three RLJ concentrations under a single cyclic-heat environment, with a separate thermoneutral group serving as a reference. Forty-eight 56-week-old Hy-Line Brown hens were housed in 16 cages of three hens. Four cages formed a thermoneutral unsupplemented reference group (C1; 25-27 °C), while four cages per concentration received 1.5% (T1), 2.0% (T2), or 3.5% (T3) RLJ under cyclic heat exposure (30-34 °C for 6 h/day) for 30 days. Cage was the experimental unit. Formal inference was restricted to T1-T3 because all heated cages shared one room or zone, whereas C1 was maintained separately. RLJ concentration was associated with differences in hen-day egg production (HDEP), egg weight, egg length, egg width, heterophil count, lymphocyte count, and heterophil-to-lymphocyte (H/L) ratio. T3 had higher HDEP than T1 and T2 (66.94% vs 53.89% and 56.11%; $P = 0.0006$). Egg weight, length, and width were greater in T3 than T1, with T2 intermediate. T3 also had fewer heterophils, more lymphocytes, and a lower H/L ratio than T1 and T2 ($P < 0.05$). Shape index, shell weight, and shell percentage did not differ among RLJ concentrations. The C1 means were presented descriptively and were not compared statistically with the heated groups. Under the realized heated-room conditions, 3.5% RLJ produced the most favorable response among the concentrations tested. However, the absence of an unsupplemented heated control and independent replication of thermal environments prevents causal conclusions about heat-stress mitigation.

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Keywords | Citrus juice, Cyclic heat exposure, Egg production, Heterophil-to-lymphocyte ratio, Late-laying hens, Rough lemon



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Introduction

Heat exposure is a major environmental constraint to poultry production, particularly in warm and tropical regions (Daghir, 2008; Liswaniso *et al.*, 2026). Chickens lack functional sweat glands and depend largely on behavioural and respiratory mechanisms to dissipate excess body heat (Lara and Rostagno, 2013). Sustained exposure to high ambient temperature can reduce feed intake and nutrient utilisation, alter endocrine and immune responses, and impair egg production and egg quality (Nawab *et al.*, 2018; Kim *et al.*, 2020).

Late-laying hens may be particularly susceptible because the nutrient demands of egg formation occur alongside age-related changes in production efficiency. Nutritional interventions that help maintain production and physiological stability during hot conditions are therefore of practical interest (He *et al.*, 2018; Abd El-Hack *et al.*, 2022). Plant-derived additives have received attention because they may supply vitamins, organic acids, phenolic compounds, and flavonoids with antioxidant or other biological activities (Saeed *et al.*, 2018).

Rough lemon (*Citrus jambhiri*) is locally available in Zambia. Its juice contains ascorbic acid, organic acids, phenolic compounds, and flavonoids. Citrus-derived products have been investigated as functional poultry additives, but responses can vary with citrus species, preparation method, concentration, palatability, physiological stage, and environmental conditions (Tavakkoli *et al.*, 2014; Saeed *et al.*, 2018). Evidence concerning graded rough lemon juice (RLJ) concentrations in the drinking water of late-laying hens exposed to cyclic heat remains limited.

A rigorous test of whether RLJ mitigates heat effects requires an unsupplemented group exposed concurrently to the same heat conditions and independent replication of the thermal environment. The present preliminary experiment did not include a 0% RLJ heated group, and the heated and thermoneutral conditions were each implemented in one room or zone. Consequently, the thermoneutral group could serve only as a descriptive reference. Formal inference was limited to the three RLJ concentrations administered to separate cages within the shared heated environment.

The study hypothesized that production, egg-quality, and leukocyte responses would differ among hens receiving 1.5%, 2.0%, or 3.5% RLJ under cyclic heat exposure. The three concentrations were selected during exploratory planning on the basis of informal preliminary observations at the study farm, in which drinking water containing up to approximately 3.5% v/v rough lemon juice was consumed without obvious refusal. These observations were not recorded systematically and did not constitute a formal palatability or dose-ranging trial. Thus, the objective was to compare hen-day egg production (HDEP), selected egg-quality characteristics, and selected leukocyte variables among these three concentrations within the realised heated environment and to describe the corresponding values observed in a separate thermoneutral unsupplemented reference group.

Materials and Methods

Study site and animal welfare statement

The experiment was conducted at Kwilanzi Farm in Chilanga District, Zambia. Rough lemon juice was characterised at the University Teaching Hospital, Lusaka, and leukocyte analyses were conducted at the School of Veterinary Medicine, University of Zambia.

Formal institutional animal-ethics review and an approval reference were not available for this experiment. Nevertheless, the hens were managed according to routine poultry husbandry and animal-welfare practices of the University of Zambia Department of Animal Science. These practices included suitable housing, unrestricted access to feed and the assigned drinking-water treatment, routine health monitoring, and careful handling to minimize avoidable stress.

Birds, treatment allocation and environmental conditions

Forty-eight 56-week-old Hy-Line Brown hens were allocated to four experimental groups. Each group comprised four battery cages with three hens per cage, giving 16 cages and 48 hens. RLJ concentration was administered by cage. Cage was used as the observational unit for production, egg-quality, and leukocyte outcomes after subsamples were averaged within cage. The retained study records did not document the procedure used to allocate treatments to cages or whether cage tier and position were incorporated into the allocation. Potential

allocation and cage-position effects therefore cannot be excluded. Each cage position remained unchanged throughout the 30-day experiment. The thermal environment was imposed at room or zone level rather than independently by cage; consequently, cages were not independent replicates of heat exposure.

The study included one thermoneutral unsupplemented reference group (C1) and three RLJ concentrations administered under cyclic heat exposure (T1-T3; Table 1). C1 was maintained in a thermoneutral room or zone, whereas all T1-T3 cages were housed in one heated room or heating zone. The three RLJ concentrations were assigned at cage level within the heated environment.

Table 1: *Experimental groups and treatment conditions.*

Group	Thermal condition	RLJ in drinking water	Hens per cage	Number of cages
C1	Thermoneutral 25-27 °C	0%	3	4
T1	Cyclic heat 30-34 °C for 6 h/day	1.5% v/v	3	4
T2	Cyclic heat 30-34 °C for 6 h/day	2.0% v/v	3	4
T3	Cyclic heat 30-34 °C for 6 h/day	3.5% v/v	3	4

RLJ = rough lemon juice.

Cyclic heat was applied daily from 10:00 to 16:00 using thermostatically controlled infrared heating lamps. C1 cages were maintained at 25-27 °C. Ambient temperature and relative humidity were monitored at bird height using calibrated HOBO U23 Pro v2 data loggers. Relative humidity during the study was approximately 55-65%. Temperature and relative humidity were monitored at room or zone level and were not recorded separately for each cage.

The thermal environments were not independently replicated: All heated cages shared one room or zone, while C1 occupied a separate thermoneutral room or zone. Thermal condition was therefore confounded with room or zone. The design also lacked a heated group receiving 0% RLJ. Consequently, the study could not provide an unbiased test of a general heat effect, an RLJ effect relative to unsupplemented heated hens, or a heat x RLJ interaction. C1 was retained only as a descriptive thermoneutral reference, and formal inference was restricted to the three RLJ

concentrations implemented within the shared heated environment.

Basal diet and feeding management

All hens received the same basal layer diet throughout the 30-day study. The diet was formulated to meet or exceed the nutrient recommendations for Hy-Line Brown hens in late lay and the relevant Zambia Bureau of Standards specifications (Hy-Line International, 2020; Zambia Bureau of Standards, 2011). Ingredient composition and calculated nutrient content of the basal diet were presented in Table 2.

Feed and the assigned drinking-water treatment were offered ad libitum. Feed and water intake were not measured. Actual RLJ intake could therefore not be calculated, and possible effects of acidity or palatability on water consumption could not be evaluated. No hens or cages were excluded because of mortality, illness, or missing observations.

Table 2: *Ingredient composition (%) and calculated nutrient content of the basal layer diet on an as-fed basis.*

Ingredient or nutrient	Inclusion or calculated value
Maize	52.20
Soybean meal	18.15
No. 3 maize meal	13.00
Fish meal	9.00
Limestone	4.00
Dicalcium phosphate	2.00
Vegetable oil	1.00
Premix	0.25
Salt	0.25
DL-methionine	0.10
Lysine	0.05
Total	100.00
Calculated nutrient content (%)	
CP	17.0
CF	4.9
Ca	4.2
Phosphorus (calculated)	0.45
ME (kcal/kg)	2,749

Preparation and characterization of rough lemon juice
Rough lemons were obtained as a single harvest from a commercial orchard near Lusaka. Fruit maturity was judged from physical appearance at harvest, but a formal maturity index was not recorded. The findings therefore describe the experimental juice

batch and should not be generalised to all *C. jambhiri* fruit, harvests, orchards, or growing conditions. Fruit number, total fruit mass, juice yield, washing or disinfection procedures, peeling and seed-removal details, operator-specific pressing procedures, mixing or homogenization procedures, and the duration of frozen storage were not documented.

The juice was extracted manually, filtered through cheesecloth with an approximate pore size below 0.5 mm, divided into aliquots in dark airtight containers, and stored at -18 °C. The quantity required each day was thawed before the drinking-water treatments were prepared. The administered material was therefore frozen-thawed RLJ from one extracted batch rather than juice freshly extracted each day.

Representative aliquots from the experimental batch were analyzed in triplicate. These measurements were technical replicates of the same juice batch and were used to describe analytical repeatability; they were not independent fruit, harvest, or juice-batch replicates. Ascorbic acid was determined using the 2,6-dichlorophenol-indophenol method. Total phenolic content was determined using the Folin-Ciocalteu method and expressed as gallic acid equivalents. Total flavonoid content was determined using the aluminium chloride method and expressed as quercetin equivalents.

Table 3: *Physicochemical and bioactive composition of the experimental rough lemon juice.*

Parameter	Mean ± SD
pH	2.3 ± 0.04
Total soluble solids (°Brix)	7.19 ± 0.15
Titrateable acidity (%)	5.31 ± 0.18
Ascorbic acid (mg/100 mL)	41.20 ± 1.45
Total phenolics (mg GAE/100 mL)	66.40 ± 2.10
Total flavonoids (mg QE/100 mL)	25.80 ± 0.95

Values are technical replicate measurements from one experimental RLJ batch and do not represent independent fruit, orchard, harvest, or juice-batch samples. Values describe the stock juice batch and not the diluted drinking-water solutions administered to the hens. GAE= gallic acid equivalents; QE = quercetin equivalents.

Drinking solutions were prepared daily from thawed stock by adding 15, 20, or 35 mL RLJ to each liter of water for T1, T2, and T3, respectively, corresponding to 1.5%, 2.0%, and 3.5% v/v. Drinkers were protected from direct sunlight. The final pH, soluble solids, titrateable acidity, and ascorbic acid concentration of the

diluted solutions were not measured. Table 3 therefore characterizes the stock juice batch rather than the solutions consumed by the hens. The concentrations were selected during exploratory planning; no formal dose-ranging, toxicity, or palatability study preceded the experiment.

Egg production and egg quality measurements

Egg production was recorded daily for each cage. Hen-day egg production (HDEP) was calculated as $HDEP (\%) = [\text{number of eggs produced} / (\text{number of live hens} \times \text{number of observation days})] \times 100$. The daily cage records were aggregated over the 30-day study to obtain one HDEP value per cage. Individual daily observations were not treated as independent replicates.

During the final week, two eggs were randomly selected from each cage, giving eight eggs per group and 32 eggs overall. Egg weight, length, width, shell weight, and shell percentage were measured. Shape index was calculated as $(\text{egg width} \div \text{egg length}) \times 100$. Egg-quality measurements were confined to the final week to provide terminal measurements after the hens had received their assigned treatments for at least 23 days. This sampling period was assumed to represent the end-of-study response; however, egg quality was not evaluated during earlier weeks, and temporal changes could not be assessed. Measurements from the two eggs were averaged within cage before analysis; eggs were subsamples, not independent experimental units.

Leukocyte assessment

On day 30, two hens were sampled from each cage, giving eight sampled hens per group and 32 hens overall. Approximately 2 mL of blood was collected from the brachial vein into an EDTA tube between 08:00 and 09:00. Morning sampling was chosen so that blood collection occurred at a consistent time of day and after overnight recovery, rather than during or immediately after the daily heat period. Thus, sampling took place approximately 16 h after the preceding heat-exposure period had ended and before the next heat period began. Two hens per cage were sampled because the cage was the experimental unit and the two hens provided a cage-level value based on two subsamples, reducing the influence of individual hen variation compared with a single hen while keeping the total number of birds handled and sampled manageable. The third hen in each cage was left undisturbed. The measurements therefore

represented leukocyte status after overnight recovery rather than an immediate response during heat exposure.

Total leukocyte counts were performed manually by an external diagnostic laboratory using a haemocytometer and its routine avian procedure, with reference to standard haematological methods (Bain *et al.*, 2017). The retained study records did not specify the haemocytometer model, blood dilution factor, diluting solution, number of squares counted, detailed counting procedure, calculation formula, number of duplicate counts performed, duplicate-count acceptance criterion or discrepancy threshold, or equipment identifiers. These details could not be inferred retrospectively, and their absence limits methodological reproducibility.

Differential leukocyte counts were conducted on Giemsa-stained peripheral blood smears at 1,000× magnification under oil immersion. For each hen, two technical counts of 100 leukocytes were made on the same smear and averaged. Differential leukocyte counts were performed by an external diagnostic laboratory following its routine avian procedure. Giemsa concentration, staining time, buffer pH, washing and drying procedures, number of slides prepared, and the exact smear region examined were part of the laboratory's internal standard operating procedure and were not disclosed to or recorded by the study team. The study team cannot therefore report these details or verify them independently. This limits the reproducibility of the differential counts. Technical repeat counts were not treated as independent biological observations.

Absolute heterophil and lymphocyte counts were calculated by multiplying the total leukocyte count by the corresponding differential proportion. The H/L ratio was calculated by dividing the absolute heterophil count by the absolute lymphocyte count. Measurements from the two sampled hens in each cage were averaged to obtain one cage-level observation. Sampled hens were therefore subsamples, and cage was the unit of analysis.

Statistical analysis

The analysis was structured to match the level at which treatments were independently applied. Because the thermoneutral and heated environments were each represented by one room or zone, room and

thermal condition could not be separated statistically. Accordingly, C1 was excluded from formal hypothesis testing against T1, T2, and T3. Its cage-level mean was presented only as a descriptive reference for the values observed in the realized thermoneutral environment.

Formal statistical analysis was restricted to T1, T2, and T3 because these RLJ concentrations were applied to separate cages housed concurrently within the same heated room or zone. HDEP was represented by one 30-day value per cage, while egg-quality and leukocyte measurements from subsampled eggs and hens were averaged within cage. Thus, each analysis included four independent cage observations per RLJ concentration and 12 observations overall. Cage was the experimental unit, and individual hens and eggs were not treated as independent replicates.

For each response, a one-factor cage-level fixed-effects model was fitted in SAS using PROC MIXED:

$$Y_{ij} = \mu + D_i + \varepsilon_{ij}$$

Where; Y_{ij} is the cage-level response for cage j receiving RLJ concentration i within the heated environment, μ is the overall mean for the heated-room observations, D_i is the fixed effect of RLJ concentration (1.5%, 2.0%, or 3.5% v/v), and ε_{ij} is the residual cage-to-cage deviation. The model assumed independent, normally distributed residuals with a common variance. The residual degrees of freedom were 9.

A formal prospective power calculation was not performed. The number of cages per concentration (four) was chosen as a practical compromise between the number of available cages, the capacity of the heated room or zone, and the number of treatment concentrations to be compared. The study was designed as an exploratory comparison, not as a confirmatory test with a pre-specified power target.

Model residuals were examined for approximate normality, homogeneity of variance, and influential observations. The overall association with RLJ concentration was assessed at $P < 0.05$. When significant, pairwise differences were evaluated using Tukey-adjusted comparisons. Least-squares means and the pooled standard error of a treatment mean were reported for T1-T3. No p value or multiple-comparison letter was assigned to C1. Statistical conclusions were therefore conditional on the single

heated environment and could not establish a general heat effect, an RLJ effect relative to unsupplemented heated hens, or a heat x RLJ interaction.

Results

Hen day egg production and egg quality characteristics

HDEP, egg weight, egg length, and egg width differed among RLJ concentrations in the shared cyclic-heat environment (Table 4). T3 had higher HDEP than T1 and T2 ($P = 0.0006$), whereas T1 and T2 did not differ. Egg weight was higher in T3 than T1 ($P = 0.0028$), with T2 intermediate. Egg length ($P = 0.0091$) and egg width ($P = 0.0312$) were also greater in T3 than T1, while T2 was intermediate.

Egg shape index ($P = 0.0854$), shell weight ($P = 0.1247$), and shell percentage ($P = 0.2183$) did not differ among RLJ concentrations. The C1 values are included in Table 4 only as descriptive thermoneutral reference means and were not compared statistically with T1-T3.

Leukocyte responses

Heterophil count, lymphocyte count, and H/L ratio differed among RLJ concentrations in the

shared heated environment (Table 5). T3 had fewer heterophils than T1 and T2 ($P = 0.0284$), more lymphocytes than T1 and T2 ($P = 0.0297$), and a lower H/L ratio than T1 and T2 ($P = 0.0315$). T1 and T2 did not differ for any leukocyte outcome.

The descriptive C1 means were numerically close to the corresponding T3 means. However, C1 was not included in the inferential model; this numerical proximity was not a test of equivalence and does not show that T3 restored heterophil count, lymphocyte count, or H/L ratio to a thermoneutral condition.

Discussion

Production and egg quality responses

Within the shared cyclic-heat environment, hens receiving 3.5% RLJ had higher HDEP than hens receiving 1.5% or 2.0% RLJ. Egg weight, length, and width were also greater at 3.5% than at 1.5% RLJ, while the 2.0% group was intermediate. The three administered concentrations were therefore not associated with the same production response under the realized heated-room conditions, and 3.5% RLJ was the most promising tested concentration for production-related outcomes.

Table 4: Hen-day egg production and selected egg-quality characteristics among hens receiving three rough lemon juice concentrations under cyclic heat exposure.

Variable	C1 descriptive reference	T1 1.5% RLJ	T2 2.0% RLJ	T3 3.5% RLJ	Pooled SEM	P-value
HDEP (%)	65.82	53.89 ^b	56.11 ^b	66.94 ^a	1.53	0.0006
Egg weight (g)	72.28	66.11 ^b	68.80 ^{ab}	71.74 ^a	0.80	0.0028
Egg length (mm)	60.22	57.57 ^b	58.90 ^{ab}	59.23 ^a	0.34	0.0091
Egg width (mm)	45.71	44.93 ^b	45.86 ^{ab}	46.35 ^a	0.25	0.0312
Shape index (%)	76.05	78.17	77.86	78.33	0.53	0.0854
Shell weight (g)	9.13	9.09	8.97	9.64	0.16	0.1247
Shell (%)	12.75	13.75	13.08	13.52	0.29	0.2183

C1 was excluded from the inferential model and carries no superscript. T1-T3 values are cage-level least-squares means from the heated-room model (four cages per concentration; residual $df = 9$). Within a row, T1-T3 means with different letters differ at Tukey-adjusted $P < 0.05$. Pooled SEM is the standard error of a T1-T3 least-squares mean. HDEP = hen-day egg production; RLJ = rough lemon juice.

Table 5: Selected leukocyte measurements among hens receiving three rough lemon juice concentrations under cyclic heat exposure.

Variable	C1 descriptive reference	T1 1.5% RLJ	T2 2.0% RLJ	T3 3.5% RLJ	Pooled SEM	P-value
Heterophils (cells/ μ L)	12,450	28,760 ^a	25,340 ^a	13,210 ^b	3,640	0.0284
Lymphocytes (cells/ μ L)	14,820	10,950 ^b	11,680 ^b	15,340 ^a	960	0.0297
H/L ratio	0.84	2.62 ^a	2.17 ^a	0.86 ^b	0.41	0.0315

C1 was excluded from the inferential model and carries no superscript. T1-T3 values are cage-level least-squares means from the heated-room model (four cages per concentration; residual $df = 9$). Within a row, T1-T3 means with different letters differ at Tukey-adjusted $p < 0.05$. Pooled SEM is the standard error of a T1-T3 least-squares mean. H/L = heterophil-to-lymphocyte ratio; RLJ = rough lemon juice. Only heterophil count, lymphocyte count, and the H/L ratio are reported. Monocytes, eosinophils, basophils, and other haematological variables were not measured.

The results do not establish that RLJ prevented or mitigated heat-related reductions in laying performance. Such a conclusion would require a 0% RLJ group exposed concurrently to the same heated environment. Because that group was absent, performance under cyclic heat without RLJ is unknown. Similarly, the descriptive proximity of the T3 and C1 means cannot be interpreted as recovery to thermoneutral performance because C1 differed in room or zone, thermal condition, and RLJ exposure.

The observed production pattern is biologically plausible. Heat exposure can reduce feed consumption, disrupt nutrient utilisation and endocrine function, and redirect nutrients away from egg formation (Lara and Rostagno, 2013; Nawab *et al.*, 2018; Kim *et al.*, 2020). RLJ contained ascorbic acid, phenolics, flavonoids, and organic acids. These constituents may contribute antioxidant or other physiological activities, and citrus-derived products have been investigated as functional poultry additives (Saeed *et al.*, 2018; Surai *et al.*, 2019; Abd El-Hack *et al.*, 2022). Studies have also examined ascorbic acid or lemon supplementation in laying hens under high ambient temperature (Ajakaiye *et al.*, 2011; Tavakkoli *et al.*, 2014).

Feed and water intake were unavailable, and the diluted drinking solutions were not chemically characterised. The quantities of RLJ and its bioactive constituents consumed by the hens are therefore unknown. Differences in palatability or water intake could also have contributed to the observed responses.

Shape index, shell weight, and shell percentage did not differ among RLJ concentrations. Thus, the production and egg-dimension responses were not accompanied by detectable changes in the measured shell traits. Shell formation may be influenced by calcium intake, acid-base balance, shell-gland function, bird age, and environmental temperature (Nuningtyas *et al.*, 2026). Because these intermediate variables were not measured and only four cages represented each concentration, the absence of significant shell responses should be interpreted cautiously.

Leukocyte responses

Hens receiving 3.5% RLJ had fewer heterophils, more lymphocytes, and a lower H/L ratio than hens receiving 1.5% or 2.0% RLJ. The combined pattern indicates a lower measured stress-associated leukocyte

response in T3 than in the two lower-concentration groups within the shared heated environment.

The H/L ratio is commonly used as an indicator of sustained physiological stress in poultry because stress-related heterophilia and lymphopenia increase the ratio (Campo and Davila, 2002; Shini *et al.*, 2008; Nwaigwe *et al.*, 2020). It is not specific to heat stress and can also be influenced by handling, infection, social stress, age, and other conditions (Khan *et al.*, 2022). The lower H/L ratio in T3 therefore supports an association between the 3.5% treatment and lower heterophil count, higher lymphocyte count, and a lower H/L ratio. The T3 leukocyte means were numerically close to the C1 reference values, but this observation is hypothesis-generating rather than evidence of equivalence or restoration. A valid test of restoration toward thermoneutral status would require independent replication of thermal environments and a design that separates thermal and RLJ effects.

Blood was collected approximately 16 h after the preceding heat period and before the next daily exposure. The results therefore reflect status after overnight recovery rather than the acute response at maximum thermal challenge. Core body temperature, respiration or panting rate, corticosterone, oxidative-damage indices, and antioxidant-enzyme activity were not measured. Including such variables at defined times before, during, and after heat exposure would provide stronger mechanistic evidence.

Scope limitations and implications

Restricting formal analysis to T1-T3 matched the treatments independently administered to cages within the shared heated environment. Averaging egg, hen, and technical-count subsamples within cage also avoided pseudoreplication. The significant differences can therefore be interpreted as cage-level differences associated with concentration, conditional on the single heated environment used in this experiment.

This analytical approach cannot overcome the design limitations. One heated room or zone does not provide independent environmental replication, and the separate C1 room prevents room effects from being distinguished from thermal effects. The missing unsupplemented heated group prevents estimation of RLJ efficacy relative to hens exposed to the same heat without supplementation and prevents estimation of a heat x RLJ interaction. In addition, the retained

records did not document the procedure used to allocate treatments to cages or whether cage tier and position were incorporated into the allocation. Potential allocation and cage-position effects therefore cannot be excluded, and any systematic relationship between cage position and treatment could contribute to the observed differences

Other limitations affect reproducibility and external validity. The study used one orchard, one harvest, and one prepared juice batch; several preparation details and the duration of frozen storage were unavailable; and the diluted solutions were not characterized. Feed and water intake were not measured, so the administered concentration could not be converted into an ingested dose. Several details of the manual leukocyte procedure were absent from the retained records. Finally, four cages per concentration provided limited precision. The study was not designed to detect a specified effect size, and the non-significant results must not be interpreted as evidence of equivalence or of no biologically relevant effect. Despite these constraints, the consistency of the response at 3.5% RLJ across HDEP, egg weight and dimensions, heterophil count, lymphocyte count, and H/L ratio provides a basis for follow-up research. The findings support prioritizing 3.5% RLJ in a rigorously controlled trial, but they do not justify recommending RLJ as an established commercial heat-stress intervention.

Significance of the study

This study provides preliminary cage-level evidence that production and leukocyte outcomes differed among three graded RLJ concentrations administered to late-laying hens within one cyclic-heat environment. Among the concentrations tested, 3.5% RLJ produced the most favourable combination of HDEP, egg dimensions, and leukocyte responses. The findings identify a locally relevant concentration for confirmatory investigation but do not establish RLJ-specific protection from heat.

Conclusion

Under the single cyclic-heat environment used in this study, hens receiving 3.5% rough lemon (*Citrus jambhiri*) juice had higher HDEP than hens receiving 1.5% or 2.0% RLJ; greater egg weight, length, and width than hens receiving 1.5% RLJ; and fewer heterophils, more lymphocytes, and a lower H/L ratio than hens receiving either lower concentration. Shape

index, shell weight, and shell percentage did not differ significantly among RLJ concentrations.

These findings identify 3.5% RLJ as the most promising of the tested concentrations within the realized heated environment. They do not demonstrate that RLJ prevented, reversed, or mitigated heat-induced effects because the experiment lacked an unsupplemented heated control and independent replication of the thermal environments. Confirmation requires an adequately powered study with replicated thermal environments, a 0% RLJ heated control, intake measurements, chemical characterization of administered solutions, direct physiological and oxidative-stress indicators, and prospective animal-ethics approval.

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Novelty Statement

This preliminary study evaluated graded concentrations of locally available rough lemon (*Citrus jambhiri*) juice in late-laying hens under cyclic heat exposure and jointly examined HDEP, selected egg-quality traits, and leukocyte responses. Its principal contribution is the identification of 3.5% RLJ as a candidate concentration for confirmatory testing within a design that transparently restricts inference to independently treated cages in the shared heated environment. The study also characterizes the experimental juice batch. It does not claim causal heat-stress mitigation because an unsupplemented heated control and independently replicated thermal environments were absent.

Author's Contribution

FK conceived and designed the study. KJS, BN, and PC conducted the experiment and collected the data. SL statistically analyzed and interpreted the data. FK drafted the manuscript. All authors critically revised the manuscript for important intellectual content and approved the final version.

Generative AI and AI assisted technology statement

The authors declare that no generative AI and AI assisted technology was used in the creation of this manuscript.

Statement of conflict of interest

The authors have declared no conflict of interest.

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